ENHANCED VACCINATION AND ANTIBIOTICS UPTAKE BY LOW INTENSITY SONOPHORESIS IN FISH

DISSERTATION

zur Erlangung des akademischen Grades

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Berlin, 2015

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This thesis is dedicated to my parents (Martín Cobo and Ana María Labarca). Thanks for all your love and support during all these three years.

CONTENTS

1 ZUSAMMENFASSUNG

Aufgrund der Überfischung der Meere verringern sich die Wildfischbestände dramatisch. Die Produktion in Aquakulturanlagen gilt als vielversprechende, nachhaltigere Alternative, um der steigenden Nachfrage nach Fischprodukten gerecht zu werden. Ein großes Problem in der Aquakultur ist das Auftreten von Fischkrankheiten und deren medikamentöse Behandlung, da dies sowohl die Anforderungen des Tierschutzes als auch des Umweltschutzes betrifft. Eine gute Strategie zur Verhinderung von Infektionskrankheiten ist die Anwendung geeigneter Präventionsmaßnahmen, insbesondere die Impfung von Fischen. Alternativ können bereits infizierte Tiere medikamentös, beispielsweise mit Antibiotika, behandelt werden. Für beiden Maßnahmen stellt sich die Frage, ob die verfügbaren Darreichungsformen für Impfstoffe und Antibiotika hinsichtlich Effizienz und Wirtschaftlichkeit verbessert werden können.

Bei Säugetieren gilt Niederfrequenz-Sonophorese (LFS) als eine der fortschrittlichsten Technologien zur lokalen, transdermalen Darreichung von Wirkstoffen. Auf dieser Grundlage entstand die Idee, bei der Impfung von Fischen im Tauchbad die Aufnahme des Impfstoffs mit Hilfe von LFS zu optimieren. Anders als bei Säugetieren, wo LFS als lokale Applikationsform auf der Haut angewendet wird, muss aus praktischen Gründen bei Fischen das ganze Individuum im Tauchbad behandelt werden.

In diesem Zusammenhang wurde in der vorliegenden Arbeit untersucht, wie sich durch eine LFS Behandlung mit einer Frequenz von 37 kHz die Aufnahme eines Bakterienimpfstoffs (formalininaktivierte *Aeromonas salmonicida*) bei Regenbogenforellen (*Oncorhynchus mykiss*) verbessern lässt und welche Nebenwirkungen bei der Behandlung auftreten. Eine Schallintensität von 171 mW/cm² reichte aus, um die Hautpermeabilität zu erhöhen. Die Fische reagierten auf die Behandlung jedoch mit unregelmäßigen, sprunghaften Schwimmbewegungen und Kiemenblutungen. Eine geringere Schallintensität von 105 mW/cm² veränderte die Hautpermeabilität nicht, war allerdings ausreichend, um während einer sechsminütigen Badbehandlung die Aufnahme des Impfstoffs in die Kiemen im Vergleich zur konventionellen Tauchbadimpfung signifikant um das fünfzehnfache zu steigern. Nach der Beschallung nahm die die Durchlässigkeit der Kiemen für den Bakterienimpfstoff

wieder ab und war nach zwei Stunden statistisch nicht mehr von derjenigen der nicht behandelten Kontrolltieren zu unterscheiden. Trotz der vielversprechenden Ergebnisse bei dieser Ultraschallintensität stellte sich angesichts der Nebenwirkungen der Beschallung auf den Fisch die Frage nach der ethischen Vertretbarkeit des Verfahrens.

Bei einer weiteren Reduzierung der Beschallungsintensität auf ~60 mW/cm² wurden nur geringe oder keine Nebenwirkungen beobachtet. Die Aufnahme des Bakterienimpfstoffs in die Kiemen während einer einminütigen Badbehandlung war allerdings immer noch um den Faktor 240 erhöht. Darüber hinaus zeigte der innerhalb von 40 Minuten nach Beschallung verringerte Albumin-Globulin-Quotient im Serum der Regenbogenforellen, dass LFS eine systemische Entzündungsreaktion auslöste. Im Vergleich zu den etablierten Impfverfahren steigerte LFS die lokale Entzündungsreaktion und die Aktivierung von T-Helferzellen in den Kiemen, was durch eine signifikant hochregulierte Genexpression von Interleukin (IL) 8, IL1ß und CD4 charakterisiert war. Die Expression der Immunglobuline IgM, IgT und IgD wurde in den Kiemen, aber nicht in der Milz und den Nieren der beschallten Fische hochreguliert. Diese Ergebnisse zeigen, dass die durch die Beschallung ausgelöste Entzündungsreaktion die mukosalen Immunreaktionen in den Kiemen anregt und Ultraschall somit Adjuvans-ähnliche Eigenschaften aufweist. Folglich hat LFS mit geringer Intensität sowohl durch die verbesserte Impfstoffaufnahme als auch Adjuvans-ähnliche Effekte das Potential, die Effektivität der Tauchbadimpfung bei Fischen ohne signifikante Nebenwirkungen zu verbessern.

Um diese Hypothese zu überprüfen, wurden Koi Karpfen (*Cyprinus carpio*) gegen das Koi-Herpesvirus und Regenbogenforellen gegen *Aeromonas salmonicida* geimpft. In beiden Versuchen erfolgte die experimentelle Infektion der Fische etwa 75 Tage nach der Impfung. Die Koi Karpfen wurden im Tauchbad mit dem Koi-Herpesvirus infiziert. Im Vergleich zu den Versuchsgruppen, die per Injektion oder im konventionellen Tauchbad geimpft worden sind, zeigte die mit LFS geimpfte-Versuchsgruppe die besten Impfresultate. Dies belegt unsere Hypothese, dass die Verwendung von Ultraschall bei der Tauchbadimpfung die Schleimhautimmunität und damit die Abwehrreaktion der Fische auf die Krankheit erhöht. Im Gegensatz zu den Karpfen wurden die Regenbogenforellen per Injektion mit *A. salmonicida* infi-

ziert. Hier stellte sich heraus, dass mit der Tauchbadimpfung mit oder ohne LFS, anders als bei Impfung per Injektion, kein Impfschutz erzielt werden konnte. Dies könnte damit erklärt werden, dass mit der unnatürlichen Infektionsroute per Injektion die Schleimhautbarriere der Kiemen umgangen wurde.

Neben der Anwendung bei der Tauchbadimpfung kann LFS auch für die Verabreichung anderer Substanzen bei Badbehandlungen verwendet werden. Als eine Möglichkeit zur Reduzierung des Einsatzes von Antibiotika in der Aquakultur untersuchten wir, wie mit LFS die Aufnahme von Antibiotika über die Kiemen verbessert und damit die therapeutische Dosis bei einer Badbehandlung verringert werden kann. Hierfür behandelten wir juvenile Regenbogenforellen mit einer geringen Ultraschallintensität (64 mW/cm² bei 37 kHz) und exponierten sie dann gegenüber verschiedenen Konzentrationen von Antibiotika, die häufig in der Aquakultur eingesetzt werden (Oxytetracyclin, Flumequin und Florfenicol). Die Resultate zeigen, dass die Ultraschallbehandlung die Aufnahme aller drei Antibiotika signifikant steigerte. Beispielsweise hatten mit Ultraschall vorbehandelte Fische nach einem Bad in 20 mg/L Oxytetracyclin sogar eine etwas höhere Konzentration des Antibiotikums in Leber und Blut als Fische, die ohne Vorbehandlung einer fünffach höhen Oxytetrazcylin-Konzentration ausgesetzt waren. Somit wäre es möglich, mithilfe LFS die therapeutische Dosis von Oxytetracyclin bei einer Badbehandlung um den Faktor fünf zu verringern und so dessen Eintrag in die aquatische Umwelt deutlich zu vermindern.

Zusammenfassend lässt sich sagen, dass LFS mit einer geringen Ultraschallintensität ein vielversprechendes Verfahren für die Aquakulturindustrie ist, da sich damit bei der Badbehandlung von Fischen die Aufnahme verschiedener Substanzen steigern lässt. Die Effektivität einer Tauchbadimpfung von Fischen kann durch die gesteigerte Impfstoffaufnahme zusammen mit der Adjuvans-ähnlichen Wirkung von niederfrequentem Ultraschall verbessert werden. Darüber hinaus ist es auch möglich, mithilfe LFS die therapeutische Dosis von Antibiotika bei Badbehandlungen zu verringern und so die medizinische Behandlung effektiver, kostengünstiger und umweltfreundlicher zu gestalten. Es erfordert jedoch noch weitere, praxisnahe Studien, um diese Technologie aus dem Labor in die Praxis zu übertragen.

2 ABSTRACT

Overfishing practices have led to a dramatic depletion of wild fish stocks. To meet the demand for fish products in a sustainable manner, aquaculture is increasingly considered to be a promising alternative source of fish. However, a major concern regarding aquaculture is the occurrence of diseases and the consequent use of chemical treatments, due to their impact on the environment, as well as on fish welfare. An effective strategy to avoid infectious diseases is to use adequate prevention methods such as vaccination of fish. Alternatively, if the fish gets infected, an option is to treat the disease with e.g., therapeutic substances such as antibiotics. Both scenarios still face the following problem: Is the way that we deliver these compounds (i.e. vaccines and antibiotics) to fish really efficient, fast, and economically feasible?

Low frequency sonophoresis (LFS) has been recognized as one of the most advanced technologies in transdermal delivery of substances in focal skin applications in mammals. Based on these findings, LFS has been suggested as a potential technology to be used for enhancement in the uptake of immersion vaccines in fish. In contrast to mammals, where LFS is applied to discrete regions of the skin, in fish the whole individual needs to be exposed for practical purposes.

In this context, the present study evaluated the impact of LFS at 37 kHz on the uptake of an *Aeromonas salmonicida* bacterin delivered via immersion to rainbow trout (*Oncorhynchus mykiss*), as well as the side effects of the treatment. The sonication intensity of 171 mW/cm² was enough to increase skin permeability, but caused heavy erratic swimming and gill haemorrhages. A lower intensity at 105 mW/cm² did not modify skin permeability, but still significantly enhanced the bacterin uptake into the gills by factor 15 during a 6 min immersion. After sonication, the permeability of the gills for the bacterin decreased, and two hours after the treatment it was statistically similar to the untreated control. However, despite these promising results, the side effects at this intensity during sonication of the fish raised some concerns as to the ethical acceptability of the procedure.

Further reduction of the sonication intensity to ~60 mW/cm² caused little or no side effects, but still increased the bacterin uptake into the gill tissue by factor 240 during a 1

min immersion. In addition, a decreasing albumin - globulin ratio in the serum of the rainbow trout after 40 min of the treatment revealed that LFS leads to a systemic inflammatory response. Compared to traditional vaccination routes, LFS boosted the local inflammatory response and T-helper cell activation in the gills, characterized by a significant up-regulation of interleukin (IL) 8, IL1ß and CD4 gene expression. The expression of immunoglobulins IgM, IgT and IgD was up-regulated in the gills, but not in the spleen or kidney of the sonicated fish. These findings highlight that the inflammatory response caused by ultrasound can boost mucosal immune responses in the gills, so that the use of ultrasound shows an adjuvant-like effect. Consequently, based on both the increased bacterin uptake and the adjuvant-like effects, low intensity LFS has the potential to improve the efficiency of immersion vaccination of fish without significant negative side effects.

To test our hypothesis, we vaccinated koi carp (*Cyprinus carpio*) against the Koi Herpes Virus (KHV) and rainbow trout against *Aeromonas salmonicida*. In both cases we experimentally infected the fish about 75 days after the vaccination. In koi carp, we infected the fish via bath immersion with the koi herpesvirus, and the group treated with ultrasound presented the highest protection (compared to injection vaccination, normal bath vaccination and control groups), confirming our hypothesis that the application of ultrasound during immersion vaccination enhances mucosal immunity and overall protection of the fish against the disease. In contrast to carp, rainbow trout were experimentally infected via injection of *A. salmonicida*. Here, it turned out that, unlike the intraperitoneal vaccination by injection, immersion vaccination either with or without LFS did not elicit protective immunity. This might be explained by the non-natural route of infection (intraperitoneal injection), which bypassed the mucosal barriers, especially of the gills.

In addition to the administration of vaccines, LFS can also be used for the delivery of other substances in bath treatments. As a possible alternative treatment aimed at reducing the use of antibiotics by the aquaculture industry, we tested the application of LFS as a method for enhancing antibiotic uptake via the gills, in this way reducing the required therapeutic dose in bath treatments. Therefore, rainbow trout juveniles were treated with low-intensity ultrasound (64 mW/cm² at 37 kHz) and then exposed to different concentrations

of three antibiotics that are commonly used in aquaculture (i.e. oxytetracycline, flumequine and florfenicol). Results show that the ultrasound treatment significantly increased uptake for all three antibiotics. As an example, fish that were pre-treated with ultrasound and then exposed to 20 mg/L oxytetracycline had even slightly higher concentrations of the antibiotic in their liver and blood than fish exposed to 100 mg/L without ultrasonic pre-treatment. Thus, LFS would allow the reduction of the therapeutic dose of oxytetracycline in bath treatments by factor five, consequently reducing its entry in aquatic environments.

In summary, LFS with low ultrasonic intensity is a promising technique for the aquaculture industry, since it can enhance the delivery of various substances during bath treatments of fish. The efficacy of the vaccination of fish by bath immersion could be improved by the increased vaccine uptake along with the adjuvant-like effect of low-frequency ultrasound. In addition, LFS could also reduce the required therapeutic dose of antibiotics in bath treatments, making them more effective, cheaper and environmentally friendly. However, further practical studies will be required to transfer this technology from the lab to the field.

3 LIST OF PAPERS

This thesis is based on three papers, which are referred to in the text by their roman numbers (I-III):

Paper I

Cobo C, Makosch K, Jung R, Kohlmann K, Knopf K. Enhanced *Aeromonas salmonicida* bacterin uptake and side effects caused by low frequency sonophoresis in rainbow trout (*Oncorhynchus mykiss*). Fish & shellfish immunology. 2014;36:444-52.

http://www.ncbi.nlm.nih.gov/pubmed/24378683

Paper II

Cobo C, Makhutu M, Lumsdon A, Thompson KD, Jung R, Kloas W, Knopf K. The adjuvant effect of low frequency ultrasound when applied with an inactivated *Aeromonas salmonicida* vaccine to rainbow trout (*Oncorhynchus mykiss*). Vaccine. 2015.

http://www.ncbi.nlm.nih.gov/pubmed/25613719

Paper III

Cobo C, Radinger J, Viehman V, Ariav R, Jung R, Thompson K.D, Kloas W, Knopf K. Application of low frequency sonophoresis and reduction of antibiotics delivered via immersion in rainbow trout (*Oncorhynchus mykiss*). Submitted manuscript (2015).

4 BACKGROUND

Aquaculture is the fastest growing sector within the food industry, providing essential nutrients, such as proteins and essential fatty acids, to some of the world's poorest regions[1]. Economically it has been estimated that hundreds of millions of families are directly or indirectly supported by this industry. In addition, some countries, such as Chile, aquaculture is also one of their most successful exports[2]. Aquaculture currently covers about 50% of the demand for aquatic products and is still increasing (Fig. 1). Among the different fish species that are cultured, rainbow trout (*Oncorhynchus mykiss*) is an "iconic" one, due to its global production (over 850 k tonnes)[3], artificially controlled lifecycle and global distribution. This species has also been widely used for research, recreational fishing and of course, as a valuable source of food.

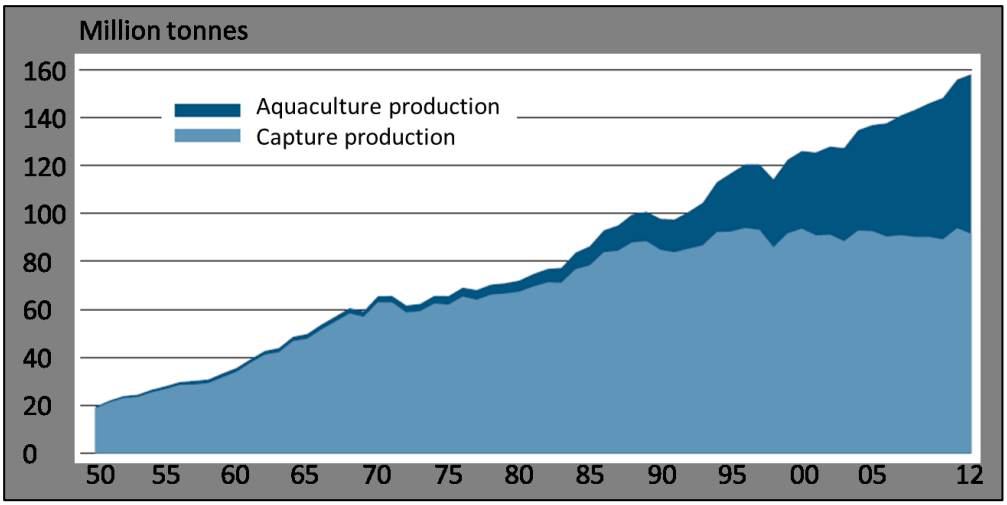

Figure 1: World capture fisheries and aquaculture production. Years are expressed in the X axis. From: FAO 2014. The State of World Fisheries and Aquaculture.

In order to improve the economical production of farmed fish, an optimization of the production line is required. This is produced by creating artificial conditions such as: confined space, pre-formulated diets, feeding schedules, selective genetic selection, human handling, controlled light regimes and higher stocking densities, among others. These factors differ from naturally occurring conditions and could disrupt normal physiological processes and stress the fish, which may result in the suppression of the immune response[4], therefore increasing the susceptibility to infection by pathogens such as viruses, bacteria, fungi, and parasites.

From an epidemiological point of view, there are three required elements (i.e.: host, habitat and pathogen) for a fish to be infected, named the disease triad. i) Host: refers to the presence of the right species for a particular pathogen. For example, koi herpes virus (KHV) will not cause disease in rainbow trout[5]. ii) Habitat/environment: pathogens also require proper environmental conditions, e.g. a bacteria such as *A.salmonicida* cannot survive in an unfavourable environment such as a 0.4% formaldehyde[6] or ozone exposures (60 seconds at 0.1 mg O_3/L)[7] iii) Pathogen: refers to any virus, bacteria, fungi and parasite that is not benign or neutral for the organism. Therefore, for disease prevention, at least one of these factors has to be removed. However, if all the three factors are present, then the presence of the pathogen in the farm is just a matter of time. Preventative action such as immunization (vaccination) should therefore be taken in order to quickly neutralize the pathogen inside the organism without causing losses to production. Currently in use in aquaculture are commercial vaccines against certain viruses and bacteria, but not parasites[8], which are mainly delivered via injection, and few of them via immersion (e.g. against Yersiniosis and Vibriosis in salmonids)[8]. The introduction of vaccines in fish aquaculture, e.g. in Norway, during the 1980's [9], drastically reduced the use of antibiotics (Fig. 2). Ideally, a vaccine should be cost effective, easy to deliver, have no side effects and immunity should last the entire production cycle.

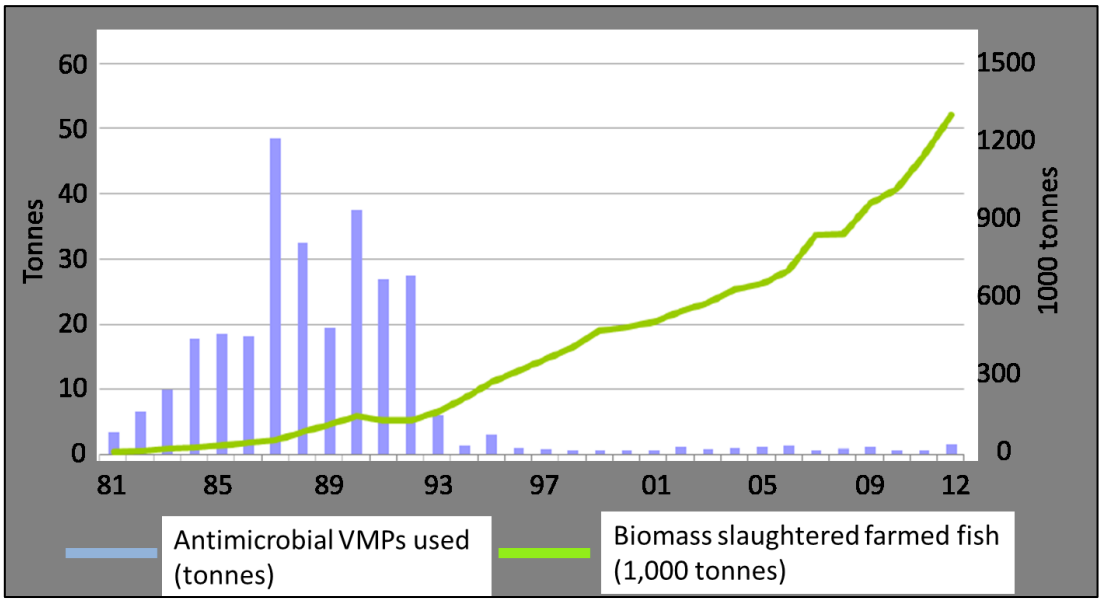

Figure 2: Total sales, in kilograms of active substance, of antimicrobial veterinary medicinal products for therapeutic use in farmed fish in Norway in the period 1981-2012 versus produced biomass (slaughtered) farmed fish. From: NORM/NORM-VET 2012. *Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway*.

Alternatively, if the fish was not immunized, for example against a bacterial pathogen, then to stop the concurrent infection an option would be to apply an antibiotic treatment to kill or inhibit the bacteria replication and stop the infection. However the use of antibiotics in aquaculture is highly criticized due to the subsequent presence of chemicals in fish and due to its contamination of the surrounding environment[10].

For the delivery of vaccines and other substances such as antibiotics there are three routes used: i) Intraperitoneal injection (IP), ii) oral vaccination and iii) bath or immersion vaccination. i) Intraperitoneal injection is considered the most efficient vaccine delivery method. IP vaccinations have constantly shown high survival rates, induced high levels of systemic antibodies, and allowed the delivery of multiple vaccines concurrently (polyvalent vaccines)[9]. Also, this method ensures that a specific dose is being delivered to each fish. However, it is a highly time-consuming task as the fish need to be anaesthetised and injected one by one, thus it is also the most expensive method[11]. ii) Oral vaccinations work by mixing the desired compound with food and therefore, unlike the IP method, this procedure

is fast and does not unnecessarily stress the animal. However, degradation of the product through the gastrointestinal tract[12] and variability of dosages are common problems associated with this method. In addition, sick fish generally stop feeding, thus inhibiting the animal from taking up medication sequestered within the feed. Lastly iii) the bath or immersion vaccination has the advantage that is easy to set up and fish are immersed into a homogenous concentration of the compound. This method is independent of fish size and can be delivered to a large mass of individuals simultaneously. However, a common problem present here is that large amounts of vaccine are required, which normally do not achieve good protection[11] despite the high doses. An additional problem is that mucosal barriers hamper entrance of substances[9], thus difficulting the uptake of vaccine.

In the present study we evaluated how low frequency sonophoresis (LFS), a modern technology for the transdermal delivery of substances in mammals [13], could overcome the problems of delivering substances via immersion in fish. Attempts at using LFS to improve the success of the immersion vaccination method were previously performed in the early 2000s with promising results[14, 15] however, despite positive outcomes, no further research was undertaken. In the present work we re-assessed and continued the work on the use of ultrasound in fish. In doing so, we realized that side effects were probably the main reason why the technique was not further studied. Therefore, we first established safe LFS parameters to work with, and then re-evaluated the technique to test whether “friendly” LFS intensities were able to enhance the uptake of substances and improve vaccination.

4.1 Low frequency ultrasound

Ultrasound has been defined as sound above 20 kHz frequency (the upper limit of the human hearing range)[16] and is commonly known for its application on image analysis for medical diagnosis (e.g. pregnancy detection). Here, the movements of small piezoelectric crystals (inside a transducer) generate sound waves of short amplitude at high frequency (between 2-5 MHz), which propagates and bounces back through a coupling media (e.g. gel) and tissues. An image then is formed by processing the return time of the sound wave[17]. This process is non-invasive and is considered to be a harmless diagnostic tool[17].

Conversely, a completely different effect can be obtained when ultrasound is emitted at low frequencies (between 20 and 100 kHz). Here, sound waves at high amplitude and low frequencies cause oscillatory changes in the atmospheric pressure of the media that is being submitted to the treatment, e.g. gel or water[18]. If a sufficient amount of energy is applied (high intensity LFS), changes in the atmospheric pressure can displace the water and create a void or cavity. This phenomenon is referred to as the cavitational effect [19, 20]. After the void's formation, it will travel at high speed towards regions of higher atmospheric pressure where it will collapse, but in the presence of a barrier such as cells/tissue, the void will violently crush and cause damage[21, 22]. This effect has not only been observed when being caused by the application of sound forces, but by mechanical forces as well e.g. the movement of a boat's propeller. In the latter example, cavitation has been extensively studied due to the occurrence of mechanical damages on industrial material surfaces[22]. This effect has also been studied for use in industrial applications like cleaning of materials and surfaces, sewage water treatment plants, and recently as a potential disinfection instrument in recirculating aquaculture systems[23].

In surfaces tissues such as the human skin, changes in permeability can be achieved by the use of LFS and increase the uptake of substances. The cavitational effect generated by ultrasound is inversely correlated to the frequency and directly related to the intensity used[24]. Consequently, the use of high frequency ultrasound (image analysis) does not cause any tissue damage or changes in permeability. Interestingly, modification of permeability in

tissues or membranes could still be achieved by non-cavitational LFS. Previous studies have observed that low intensity LFS causes oscillatory movements in membranes, including the lipid by-layer membrane[25], which leads to an increase in permeability by the formation of aqueous channels. However if intensities are higher it will led to membrane disruption. The magnitude or intensity of these oscillatory movements can be drastically reduced if the exposed membrane or cell layer is embedded in or supported by other tissues [25].

Application of LFS on mammals skin and its effect on the stratum corneum has been studied since the mid 1990´s [26].Low frequency ultrasound creates aqueous pores that facilitates and enhances the uptake of particles such as manitol, estradiol, aspirin and others [27]. Even larger molecules such as antigens (vaccines) have been delivered through mammalian skin via LFS without any detrimental side effects[28], thus suggesting that LFS is a suitable delivery system for medicinal substances. Pioneering work on the utilization of LFS in fish started over a decade ago. Previous studies described that local application of ultrasound to goldfish skin caused lysis of the outer epithelia[21] and an increased uptake of AgCl particles [29]. Furthermore, authors reported an increased uptake of larger molecules (bovine serum albumin) by applying a local sonication to the skin of goldfish [30]. Other pioneering work included inducing increased protection in trout subsequently infected with the Viral Hemorrhagic Septicemia virus when trout were previously immersed, vaccinated and sonicated (400 mW/cm^2)[15], and full protection against *Vibrio alginolyticus* in grouper (*Epinephelus awoara*)[31] and sea bream (*Pagrus major*)[14] when immersed and sonicated at ~250 mW/cm^2. However, Fernandez-Alonso *et al.*[15] reported that the total duration of ultrasound exposure was about half of that required for it to have deleterious observable effects, while Zhou *et al*.[31] stated that intensities above 400 mW/cm^2 can even cause mortality rates in fish.

As was previously mentioned, the transdermal delivery of substances enhanced by ultrasound was originally studied in mammals. Here, sonication was applied to a confined section of the skin and therefore intensities and side effects were directly studied over that specific surface. For example, in rats, it was shown that ultrasound intensities of about 2.4 W/cm² cause slight erythema on the body skin. However, when applied specifically to the

head at 0.5 W/cm², the treatment caused brain edema [32]. Conversely, if the delivery of substances is planned to be administered to a large number of individuals, as is the case with fish in aquaculture, sonication of the whole individual is necessary for practical reasons. Consequently, and unlike in mammals, the applied intensity has to be tolerable for all of the external parts of a fish and not only a single pre-defined area. Also important to bear in mind is that in fish, the potential impairment of the permeability of the outer membranes caused by LFS could be a route of entrance for aquatic microbes; this aspect also needs to be considered in the evaluation of the side effects of LFS in aquatic organisms.

4.2 Fish Immune system

In order to understand the immune system and how vaccination works in fish, it is better to split the immune system into i) innate (natural) and ii) adaptive immune systems. Due to integration of their components, clear differences between these two systems are not always clear. The natural immune system is characterized by non-specific, low and acute immune response that includes physical barriers, cellular and humoral components[33]. On the other hand, the adaptive immune system is characterized by a specific, high and chronic immune response mediated by humoral and cellular components, specifically antibodies and lymphocytes[34]. Lymphocytes are specialized cells able to memorize an antigen and produce antibodies, which will specifically bind and neutralize pathogens that possess specific antigens.

The innate immune system

The innate immune system is commonly referred to as the first line of protection when a pathogen or an unknown type of structure (metals, chemicals, proteins, carbohydrates, cells, virus, bacteria, and parasites among others) tries to penetrate the organism. In fish, the first outside barrier for pathogens is the mucous layer, which is actively secreted by mucous or Goblet cells[35]. Goblet cells are concentrated in the epidermis of the skin, gill epithelium and through almost the entire gastrointestinal tract[36]. Mucous has bactericidal properties[37] and can mechanically remove particles which attach to the skin. This offers a high resistance to pathogens but also prevents absorption of potentially beneficial substances, such as vaccines and antibiotics.

Gills are generally considered the most important and unprotected physiological organ in fish[38]. They are responsible for the gas interchange, metabolic waste excretion in the form of ammonia (unlike mammals, which is excreted via conversion to urea and secreted in urine) and ion balance with the Na/K ATPase[39]. Due to its respiratory functions, gills are only covered by a single layer of epithelial cells to allow gas diffusion, probably resulting in a lower resistance against the entrance of pathogens compared to the other external barriers (i.e. the gut and the skin). Therefore, mucosal immunity in the gills is of vital importance in

avoiding the entrance of pathogens. In terms of protection, Haugarvoll *et al.* (2003) discovered that gills possess a unique lymphoid tissue[40]. Additionally, Zhang *et al.* [41, 42] and Hansen *et al.*[43] observed the presence of a specific mucosal antibody within the gills of rainbow trout. These studies suggest that gills are not only protected by innate immune responses, but that this external organ could also respond with an adaptive immune response for a more efficient defence against pathogens.

When a pathogen (e.g. bacteria) enters into the body, it will be recognized by the immune system via pathogen recognition receptors located in various type of cells, like macrophages. These cells will detect pathogen associated molecular patterns, e.g. lipopolysaccharides, located in the outer membrane of the bacteria[44, 45]. This recognition will induce phagocytosis, which results in macrophages engulfing foreign organisms. By doing this, the macrophage will also i) degrade the pathogen and secrete part of it to its outer membrane and ii) secrete humoral factors such as cytokines (regulators of the immune system) like interleukins (e.g. IL 1ß), interferons and complement factors for the attraction of other components of the immune system[46, 47]. Also, in the presence of foreign bodies epithelial cells will secrete chemotactic factors, such as IL 8, that will summon immune components such as macrophages[45]. The presence of these cytokines (IL 1ß and Il 8) is characteristic for an inflammatory response which is a desirable reaction for antigen presentation and the induction of the adaptive immune response[48].

The adaptive immune system

In terms of evolution, fish are one of the most ancient organisms to have evolved specialized cells called lymphocytes, which are major cellular components of the adaptive immune response[49]. These cells are able to recognize, memorize, and produce specific antibodies against pathogens. This allows the immune system to quickly recognize pathogens which re-enter an organism, and mount a more specific and efficient response mediated by antibodies.

Lymphocytes are divided into B and T cells, depending upon the type of receptors they possess. B cells are produced in primarily lymphoid organs, such as the head kidney, but also can be found in blood, spleen (white pulp) and the gut[41, 46]. B cells can be summoned in the organism by lymphokines, e.g. IL-1ß (produced by macrophages).

Antibodies (Ab) are specialized proteins produced by plasma cells, which can specifically bind to different epitopes (antigenic determinants). When an Ab is expressed on the membrane of a B cell, it can recognize epitopes in the membrane of a pathogen and engulf them[33, 45]. After engulfment, the epitope will be processed and carried out to the cell membrane bounded to a glycoprotein of the B cell[46]. This binding glycoprotein is known as the Major Histocompatibility Complex Class II (MHC II). MHC Class II also can be codified by macrophages and dendritic-like cells; due to this function these cells are also named antigen presenting cells. The B cell that has recognized and engulfed a pathogen will be stimulated to secrete humoral factors that can i) stimulate the differentiation of B cells into plasma cells (also called antibody secreting cells), ii) cytokine production, iii) complement activation and iv) promotes phagocytosis[46, 50]

As previously mentioned, another type of lymphocyte is the T cell. These are mainly produced in the thymus and can be sub-divided into cytotoxic T cells (Tc) and helper T cells (Th). Tc have a protein receptor called CD8 in their membrane, which can bind to a membrane glycoprotein called MHC Class I, which also serves as co-receptor for TCRs[46]. Previous studies have identified the presence of the MHC I component in the salmonid genus (*Salmo sp.*)[51]. A MHC Class I protein is present on the surface of all nucleated cells, and it can be expressed when the cell is injured. With the expression of the MHC I glycoprotein, a Tc will bind through its CD8 receptor and cause cell lysis; normally this type of response occurs in viral infections[52]. The other type of T cell is the Th cell, which contains a specific membrane protein receptor called CD4. This protein binds with the MHC class II protein, expressed in B cells, macrophages and dendritic cells. The binding of an MHC class II, expressed by macrophages, with a CD4 receptor in the lymphocyte, combined with the co-stimulation of B cells

by cytokines released by activated Th cells will trigger the differentiation of B cells into antibody secreting cells (plasma cells) and memory B cells.

To date, only 3 types of antibodies (immunoglobulins) have been described: IgM, IgT and IgD in fish[42]. IgM has been through years the only antibody that could be used for the evaluation of the adaptive immune response in fish[53]. For example, increased levels of IgM in serum have been positively correlated with higher percentage of survival in fish vaccinated and afterwards infected *e.g. A.salmonicida* [54]. Nevertheless, it has been reported that high levels of antibody titers after vaccination are not always correlated with protection against *A.salmonicida*[55], suggesting that IgM levels in serum are not always a reliable indicator of protection against disease. With regard to mucosal immunity, the latest antibody to be discovered in fish was IgT by Hansen *et al*. (2005)[43]. This discovery expands the potential research areas regarding the adaptive immune response of fish. The presence of IgT covering the epithelium of mucosal organs was found in rainbow trout, experimentally infected with *Ceratomyxa shasta*[41] and later a significant up regulation of IgT in the gills of trout experimentally infected with *Ichthyophthirius multifilis*[56], suggesting the local production of IgT. The presence of IgT plus the discovery of a lymphoid tissue on the gills shows that local gill immune response via innate barriers and antibodies could be sufficient to achieve protection. This may also explain why low serum IgM levels in fish are not necessarily a good determinant of protection from pathogenic organisms. Specific functions for IgD are still not clear and only few reports are available, however the presence of IgD in rainbow trout[57] and its potential role in protection of respiratory mucosa[58] has been recently studied.

As previously described, the discovery of localized lymphoid tissues on the gills, plus the presence of a specific mucosal antibody suggest that pathogens can be quickly detected and neutralized on external barriers. Therefore, the induction or triggering of an adaptive immune response on mucosal surfaces is a highly desirable for vaccination. A vaccines function is to deliver pathogenically inactive antigens for recognition and processing by the immune system. Low frequency ultra-sound may enhance delivery of antigens by oscillatory movements on mucosal surfaces, plus the presence of the pathogen could elicit a stronger

immune response in this area. Previous studies have shown that the presence of adjuvants on vaccines greatly improves protection by triggering an inflammatory response and helping in the persistence of the antigen in the organism for a longer period of time[59]. As previously described, Norway greatly reduced the use of antibiotics after the introduction of vaccination; specifically after the introduction of oil adjuvants in a vaccine against *A.salmonicida* [60]. Therefore, we hypothesized that the LFS presents a great potential to act as an adjuvant and will assist in triggering the adaptive immunity. Compared to other treatments, ultrasound increases the uptake of vaccines with only minimal side effects[61].

4.3 Antibiotics treatments

When fish are subjected to stressful conditions the likelihood for a diseases appearance and epidemiological outbreaks is higher. Diseases in aquaculture are one of the greatest challenges that fish producers face nowadays[62]. For example, septicemic ricketsial syndrome[63] (SRS, caused by *Piscirickettsia salmonis*) caused loses of $400 million[63] in Chilean aquaculture in 2006. Furthermore, in Thailand, more than $1 billion were lost due to sanitary problems[64] between 2012 and 2013. In addition to disease prevention via good farming practices, one potential solution for an aquaculture company which has an ongoing infection in its farm is to treat the fish with chemical therapies, e.g. antibiotics. The use of chemical therapies is still commonly practiced in many countries during a disease outbreak. However, the use of antibiotics in aquaculture is highly variable between different regions.

Reliable estimates regarding total quantities of antibiotics used in aquaculture are hard to obtain. Current information derives from reported cases of environmental contamination[65, 66], official governmental reports made by some countries[67, 68] and indirect estimations[62]. For example, in Norwegian salmonid aquaculture, it has been reported that the amount of antibiotics used is less than 1 g per ton of salmon product. By contrast, over 150 g is used in Canada and over 1000 g per ton in Chile[69]. It has been indirectly estimated that Vietnam uses more than 700 g antibiotics per ton of aquaculture product[62]. For China, the biggest aquaculture producer in the world, there is no data available regarding the quantity of antibiotics being used in aquaculture. However, reports point out high concentrations of antibiotics in the effluents of fish farms[70], while thousands of fish are being rejected for export to the USA due to high concentrations of antibiotics[71]. In Asia at least 36 different types of antibiotics are being applied to farmed fish during various stages of their lifecycle[72]. Common antibiotics being used globally include flumequine, florfenicol and oxytetracycline, the latter being considered the most commonly used antibiotic in aquaculture[73].

High concentrations of antibiotics not only “contaminate” the fish, but also the surrounding environment, causing toxicity on primary producers and changes in the assemblage of microbial communities and chemical contamination on natural systems. Therefore,

the use of antibiotics in aquaculture should be stopped. However, that is an unreal scenario since the presence/risk of diseases cannot be eliminated. Consequently antibiotics will always be the primary method for treatment of bacterial diseases in fish.

The application of LFS in mammals has been shown to increase the transdermal uptake of several substances[26]. In fish, the benefits of this technique have only been assessed for vaccination purposes. Due to the environmental relevance and high impact of this topic, we studied the use of LFS for the uptake of oxytetracycline, flumequine and florfenicol given at two different concentrations each with and without ultrasound. Here we hypothesized that low intensity low frequency ultrasound could enhance the uptake of antibiotics in rainbow trout and therefore reduce the quantities used. When information regarding the use of antibiotics and route of delivery is hard to obtain or unavailable then is difficult to make an accurate estimation in order to assess the potential reduction of antibiotics that could be avoided with the use of LFS. Chile is one of the few well documented examples. For example, based on the last official data of 2013[68], it is known that 8.4 tons of oxytetracycline was used to treat Flavobacteriosis (caused by *Flavobacterium sp*.) in the freshwater stage in salmonids in that year. Also, there is an official handbook from the same country that recommends the use of oxytetracycline by bath for Flavobacteriosis[74]. This indicates that immersion antibiotics are still in use and even small changes in the uptake of this substance would result in a decrease in the amount of antibiotics required.

5 AIMS OF THIS STUDY

Aims of the 1st study

- Investigate the effects of low frequency ultrasound intensity on fish and identify an appropriate level that can be applied to the whole fish with minimal or no side effects.
- Assess whether this intensity can increase permeability or not.
- Determine, in the case of increased permeability, how long does this last.

These aims were fully covered in the -Paper I- of this thesis, entitled "Enhanced *Aeromonas salmonicida* bacterin uptake and side effects caused by low frequency sonophoresis in rainbow trout (*Oncorhynchus mykiss*)"

Aims of the 2nd study

- Assess whether low intensity LFS can induce activation of key immune components, such as IL 1ß and IL 8.
- Check if the presence of ultrasound assists in the antigen persistence inside the fish.
- Compare the adaptive immune response in fish vaccinated with and without sonication.

These aims were studied in detail in the -Paper II- of this thesis, entitled "The adjuvant effect of low frequency ultrasound when applied with an inactivated *Aeromonas salmonicida* vaccine to rainbow trout (*Oncorhynchus mykiss*)"

Aims of the 3rd study

- Investigate whether low intensity low frequency ultrasound improves the uptake of oxytetracycline, flumequine and/or florfenicol in rainbow trout.
- Estimate the magnitude of this uptake.

These aims were assessed in detail in the -Paper III- of this thesis, entitled "Application of low frequency sonophoresis and reduction of antibiotics delivered via immersion in rainbow trout (*Oncorhynchus mykiss*)".

6 MAIN MATERIALS AND METHODS

The present project focused on the novel application of low frequency ultrasound on fish. For this we worked in co-operation with an industrial partner that has 20 years' experience in the use of ultrasound, BANDELIN electronic GmbH & Co. KG. The company designed an ultrasound prototype specially manufactured for use with fish (Papers I, II and III). This device has a 17 (Fig. 3) litre inner basin of 10 cm width and is equipped with 20 transducers in each lateral wall. The apparatus has a fixed low frequency ultrasound of 37 kHz and intensities can be adjusted in a range from 26 to 540 mW/cm². For the sonication trials, intensities were calculated using the total power consumption (measured in watts) divided by the area encompassed by the transducers and corrected with an efficiency factor provided by the manufacturer. Thus, calculations of the intensities in the present work refer to intensities emitted from the transducers and not measured in the water media. Detail of this calculation is described in Paper I and a diagram of the device can be found in Paper III. Sonication was performed in pulses of 30 seconds with the device ON/OFF as described by Zhou[31], and substances were administered during or after the sonication procedure. We worked with rainbow trout of a maximum of 70 g (Paper I, II and III) and koi carp (*Cyprinus carpio*) of ~20 g (unpublished data) as a model species.

Figure 3: Picture of the ultrasound device used in the present study fill with 17 litres water and rainbow trout juveniles (~10 g).

Assessment of the side effects caused by ultrasound were analysed by: i), macroscopic observations during and after the application of ultrasound, ii), histological section analysis and iii) by changes of pH and levels of Na^{+}, K^{+}, Ca^{++}, Mg^{++}, Cl^{-}, inorganic phosphate, and proteins in the blood (Paper I).

The key component of our methodology was to assess how LFS can change the permeability of the skin and gills to external substances. To achieve this we developed a fast and reliable method for the relative quantification of a bacterial vaccine in tissues, which is described in Paper I and repeated on Paper II. In brief, the bacterium *Aeromonas salmonicida* was cultured in trypticase soy agar and then inactivated by formalin at 0.4%. After the inactivation bacterin was administrated to the fish via bath (with or without ultrasound) or injection and samples of different organs (e.g. gills) were taken and preserved in 99.8% ethanol. Subsequently, DNA was extracted and the relative quantification of the bacterial

DNA was performed by quantitative polymerase chain reaction (qPCR). Primers used were taken from the literature[75] and the identity of the PCR product was confirmed by sequencing of the PCR product (Paper I). For endorsement of the bacterin uptake, image analyses were performed (Paper I), by staining histological sections with monoclonal antibodies against *A. salmonicida* (Immunohistochemistry).

Furthermore, the effectiveness of low-frequency sonophoresis in fish was assessed for the application of antibiotics (i.e.: flumequine, oxytetracycline and florfenicol) by immersion. Consequently, the concentrations of the antibiotics in serum and tissue samples were determined by means of ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry (UPLC/Q-TOF-MS) (Paper III).

The relative gene expression of key immune components such as Interleukin (IL) 8, IL 1ß, cluster of differentiation (CD) 4, CD 8, class I major histocompatibility complex (MHC I), MHC II, immunoglobulin (Ig) M, IgT and IgD was quantified by qPCR. Serum antibody levels were determined by an enzyme-linked immunosorbent assay (ELISA) (Paper II) and total serum globulin levels were also analysed (Paper I).

7 MAIN RESULTS

Side effects (Paper I)

Treatment of rainbow trout at LFS intensities of 171 mW/cm^2 revealed deleterious side effects such as bleeding through the gills, exophthalmia and erratic swimming, amongst others. Lower intensities such as 105 mW/cm^2 also induced erratic swimming as a visible side effect. Intensities of ~60 mW/cm^2 caused only occasional uncoordinated movements. Intensities of 26 mW/cm^2 did not induce any visible side effects. Histological evaluation revealed severe changes in the gills of fish that were treated with ultrasound at 105 mW/cm^2. In contrast, LFS intensities of ~60 mW/cm^2 or below did not cause such deleterious changes, and were therefore chosen as safe intensities to work with.

Changes in permeability (Paper I, II and III)

Fish that were exposed to ultrasound and the bacterin vaccine (inactivated *A.salmonicida*) showed that the increased permeability in the gills directly depends on the sonication intensity (Paper I). Changes to the permeability of the skin and gills were induced at intensities of 171 mW/cm^2, but intensities of 105 mW/cm^2 or below only modified the permeability of the gills but not the skin (Paper I). Bath immersion of the vaccine in fish sonicated at ~60 mW/cm^2 resulted in a significant increase (240 fold higher) in the permeability of the gills (Fig. 4) compared to the group that did not receive the sonication treatment (Paper II). In terms of the reversibility of the effects caused by LFS, we examined the duration of the effect of increased permeability. Measurement of the bacterin uptake after sonication with an intensity of 105 mW/cm^2 showed that up to 20 minutes later the permeability of the gills was still increased, but this increased permeability was no longer detected two hours after the treatment (Paper I).

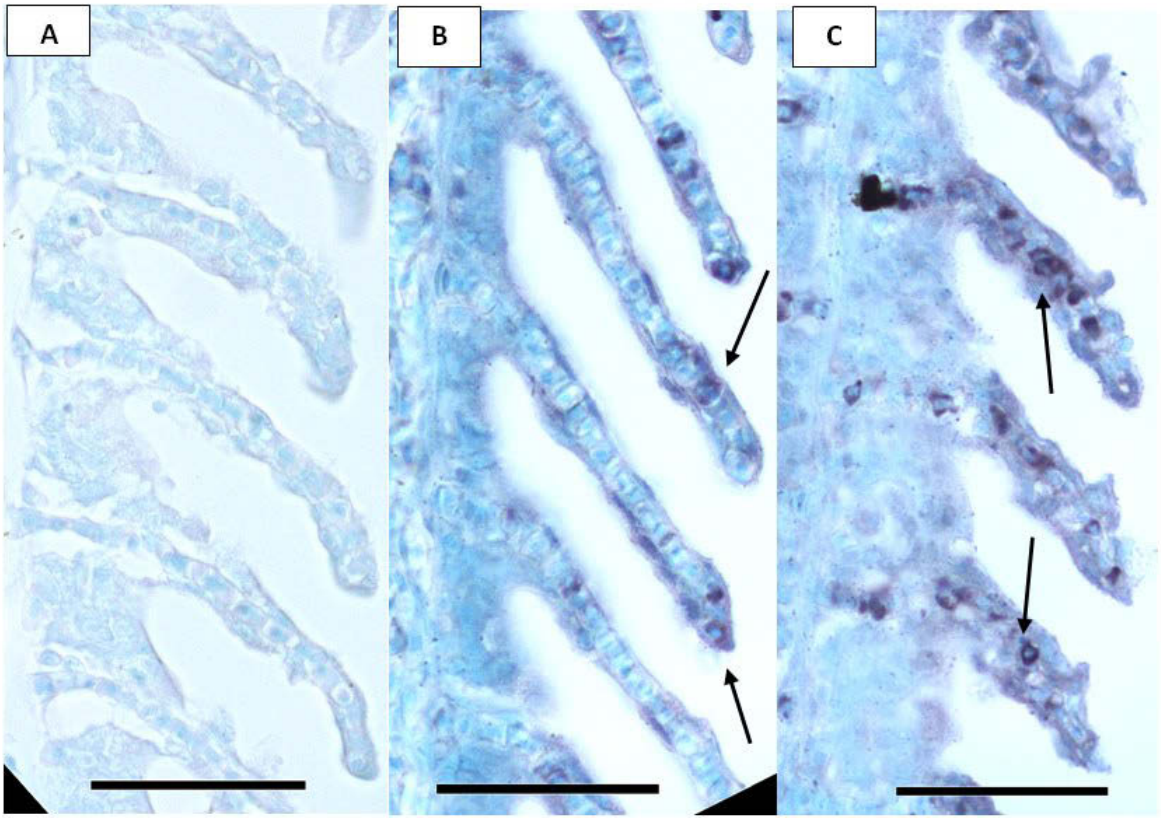

Figure 4. Immunohistochemical stained sections with monoclonal antibodies against *Aeromonas salmonicida* in rainbow trout. A) Gills of not exposed control fish , B) Gills of fish exposed to the bacterin. C) Gills of fish exposed to ultrasound (37 kHz, 105 mW/cm², 5 pulses of 30 s) and the bacterin. Solid arrow indicates marked bacterin. Bar indicates 50 µm. From: Cobo *et al.* 2014. Enhanced *Aeromonas salmonicida* bacterin uptake and side effects caused by low frequency sonophoresis in rainbow trout (*Oncorhynchus mykiss*).

In the assessment of the uptake of antibiotics, results showed that LFS significantly enhanced the uptake for flumequine, oxytetracycline and florfenicol (Paper III). Preliminary results in fish immersed for six min in flumequine (with and without ultrasound) at 20 mg/L revealed that ultrasound increases the uptake of this substance, showing mean concentrations of 126 and 47 ng/mg respectively in liver (n=7) (unpublished data). However, this time of exposure is unrealistic when compared to commercial practices. Consequently, we exposed fish for one hour to antibiotics dissolved in water with and without ultrasound treatment (Paper III). Here, for example, we found that for flumequine, concentrations in liver

were 1394 and 851 ng/mg (fish treated with and without ultrasound respectively). Also, it was found that LFS increased the uptake of oxytetracycline (Fig. 5) by factor 5. For florfenicol, LFS also increased the uptake significantly, but we could not estimate to which magnitude (Paper III).

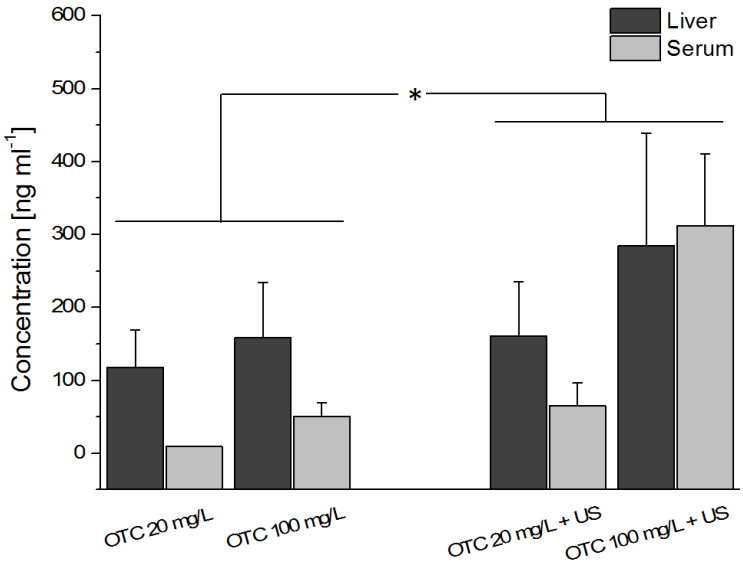

Figure 5: Mean concentration and standard deviations (n=7) of oxytetracycline (OTC) in the liver and serum of rainbow trout after exposure to OTC at 20 mg L^{-1} or 100 mg L^{-1} by immersion with or without ultrasound (US). An Anova significant difference is marked by an asterisk. From: Cobo C, Radinger J, Viehman V, Ariav R, Jung R, Thompson K.D, Kloas W, Knopf. 2015. Application of low frequency sonophoresis and reduction of antibiotics delivered via immersion in rainbow trout (*Oncorhynchus mykiss*) (submitted manuscript)

Effects of LFS on the innate and adaptive immune responses (Paper I and II)

Fish that were exposed to ultrasound at ~60 mW/cm² but not vaccinated experienced a significant increase of the total serum globulin 40 min after sonication (Paper I). Further research on inflammatory proteins revealed a significant upregulation of IL 8 and IL 1ß in the gills 6 h after a LFS-assisted immersion vaccination (Paper II). Similarly, analysis of the gills 35 days after vaccination revealed an upregulation of IgM, IgD and IgT (the first two being statically significant) in fish that were vaccinated with ultrasound assistance (Fig. 6),

whilst fish that were bath vaccinated without ultrasound or vaccinated via the intraperitoneal injection (IP) route showed a down regulation of the genes mentioned (Paper II).

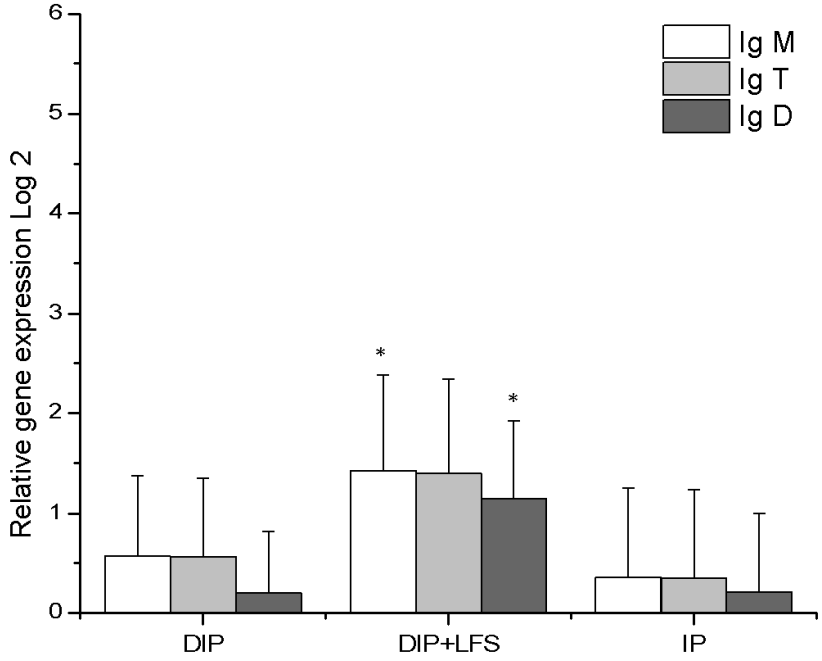

Figure 6: Expression levels of Ig M, T and D in the gill of rainbow trout 35 days after vaccination with an *Aeromonas salmonicida* bacterin. Relative gene expression to the housekeeping gene EF1. Abbreviations: Control – control group; DIP- dip vaccinated group; DIP+LFS – group dip vaccinated with ultrasound; IP – intraperitoneally injected group. Values represent the average and standard deviation of seven samples. Asterisk indicates significant differences compared to the control. From: Cobo *et al.* 2015. The adjuvant effect of low frequency ultrasound when applied with an inactivated *Aeromonas salmonicida* vaccine to rainbow trout (*Oncorhynchus mykiss*). *Vaccine* (In press) [76]

Finally we performed two infection trials (unpublished data). Here, fish were split into the following groups: i) normal immersion, ii), LFS-assisted immersion with, iii), IP vaccination and iv), control. In the first trial we used *A. salmonicida* in rainbow trout, and for the second Koi Herpes Virus in koi carp. In both trials, challenge infections were performed about 70 days after the vaccination. The route of infection varied for each experiment due to viability of the established infection model. For rainbow trout the infection was per-

formed via IP injection and for koi carp via immersion. Results show that for the rainbow trout trial, the immersed vaccinated groups (with or without ultrasound) did not elicit any protection, showing the highest mortalities (Fig. 7). In contrast, for koi carp the application of LFS improved the efficiency of the immersion vaccination against KHV and led to higher protection compared to normal immersion, IP vaccination and control groups. (Fig. 8)

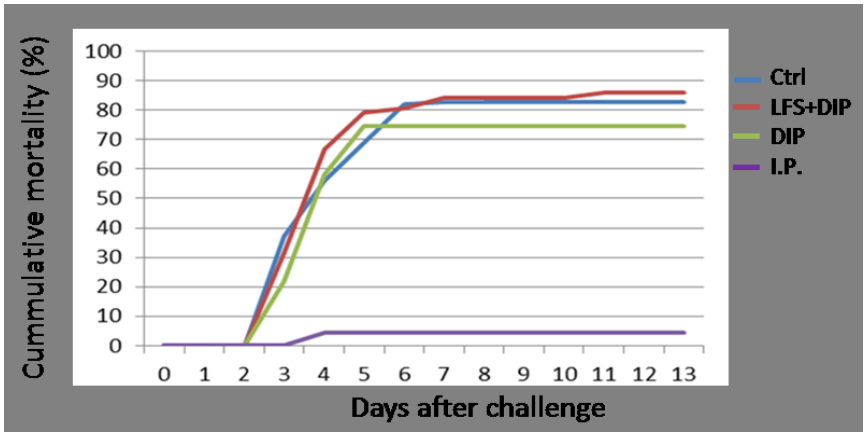

Figure 7: Vaccination trial against Furunculosis. Cumulative mortalities in rainbow trout after infection via intraperitoneal injection with *Aeromonas salmonicida* (n=40 per group). Ctrl = without immunization; LFS+DIP = immunization via bath with ultrasound; DIP = only bath immunization; I.P. = immunization via intraperitoneal injection.

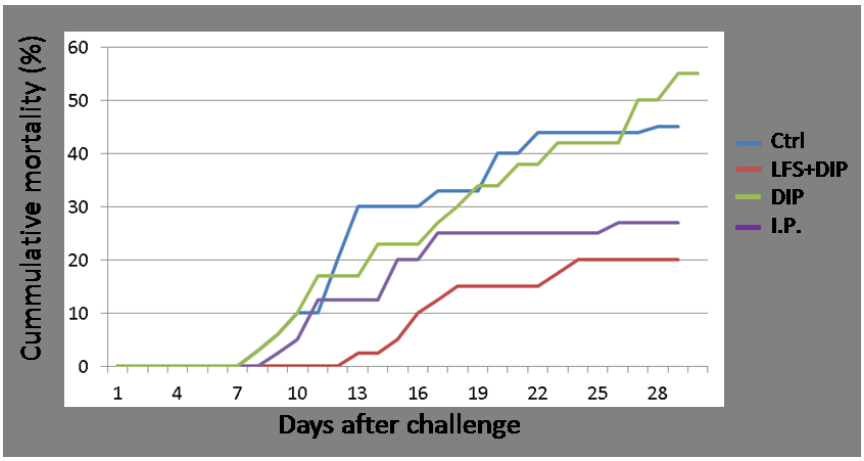

Figure 8: Vaccination trial against KHV. Cumulative mortalities from koi fish after infection via bath immersion for one hour with Koi Herpes Virus (n=35 per group). Ctrl = without immunization; LFS+DIP = immunization via bath with ultrasound; DIP = only bath immunization; I.P. = immunization via intraperitoneal injection.

8 GENERAL DISCUSSION AND CONCLUSIONS

In the present study we analysed the use of low intensity LFS as a potential technology for a more efficient delivery of substances by immersion in aquaculture. The LFS-assisted immersion was compared to normal immersion and injection routes. Pioneer works using LFS at medium or high ultrasonic intensities in fish explored the enhancement in delivery and protection achieved by immersion vaccines. Promising results in terms of increased vaccine uptake and relative percentage of survival in vaccination trials[14, 15, 31, 77] were revealed. However, in previous works, assessments of the side effects caused by sonication were almost not considered.

With regard to the safety of the technique, our examinations performed with ultrasound at 37 kHz showed that the side effects and uptake of the vaccine were proportionally related to the ultrasonic intensity (ranging from 25 to 171 mW/cm²). On this basis, we conclude that the best compromise between fish welfare and increased uptake is at intensities of ~60 mW/cm², which is approximately 2 to 10 fold lower than the intensities applied for the transdermal delivery of substances in mammals [26, 78] (Paper I). The vulnerability of the fish to ultrasound intensities which are successfully used in mammals can be explained in particular by the morphological features of the fish skin, having a stratified squamous epithelium instead of the outer keratinized epithelial layer. Furthermore, in fish, sonication of the whole individual not only affects the skin, but also simultaneously affects other tissues that are more fragile such as the gills.

Why is the gill epithelium more fragile than the skin epithelium when submitted to the LFS effect? Recently, a hypothesis was published that explains that non-cavitational ultrasound causes oscillatory movements of free membranes. These oscillatory movements are considerably attenuated by the presence of surrounding or supporting tissue[25]. In contrast to the skin, the gill lamellae are only covered by a simple epithelium composed of only a single layer of cells plus its basal membrane[38], which explains why the gill epithelium was more fragile than the skin when treated with ultrasound (Paper I).

Although one of the aims of this study was to increase permeability of the gills and so facilitate the uptake of vaccines or antibiotics by means of an LFS treatment, the same mechanism potentially provides a route of entry for undesirable particles, e.g. pathogenic bacteria. According to our results, the increased permeability of the gills returns to the original state within 120 min after sonication with an intensity of 105 mw/cm², then it must be concluded that the water quality parameters of the fish holding waters during the first two hours after the sonication should be maintained at the highest standards possible (Paper I). For lower ultrasound intensities such as ~60 mW/cm² (which we recommend as a suitable intensity at which to work), the period of time required for the recovery of the normal permeability status may be shorter; however, this was not assessed.

As studied in mammals, vaccination assisted by LFS does not only improve the uptake of the vaccine, but also acts as an adjuvant improving the immune response through the activation of antigen presenting cells. Accordingly, we found that in fish the initial inflammatory response caused by the application of LFS (alone or accompanied by a vaccine) also acts as an adjuvant on the gills (Paper II). Here, we focussed our study on the gills since i), ultrasound has a greater effect on them compared to the skin (Paper I), and ii), the immune response of the external barriers is critical as a first line of defence against pathogens such as bacteria. In the gills, LFS caused the release of a strong chemotactic factor, IL 8, along with the activation of macrophages (characterized by the up regulation of IL 1ß). Both chemokines triggered the adaptive immune response in the presence of the antigen, characterized by the later up regulation of IgM, IgT and IgD in the gills of fish that were vaccinated with the assistance of LFS. Remarkably, only fish groups that were not exposed to ultrasound (i.e. bath and intraperitoneal vaccinated) showed a significant upregulation of these immunoglobulins (antibodies) in the spleen. This finding gives strong evidence on the role of LFS as a mucosal adjuvant in fish (Paper II).

Subsequently, we tested the potential adjuvant effect of LFS in a more real scenario by vaccinating the fish and infecting them ~70 days after the vaccination (unpublished results). In koi carp that were vaccinated against KHV, the group that was vaccinated via im-

mersion with ultrasound assistance showed the lowest mortality after a KHV infection-challenge by "mucosal" administration of the virus (i.e. immersion infection). Although the sample size of this pilot study was too small to obtain statistical evidence, these results strongly suggest that the application of LFS can considerably improve the efficiency of conventional immersion vaccination. In contrast, in rainbow trout we failed to show that immersion vaccination against *A. salmonicida* (with or without ultrasound) induces protective immunity when applying an infection-challenge by IP injection of the bacteria (data not shown). The high mortality of fish vaccinated by bath immersion could be explained by the different routes of vaccination and infection. One might assume that immersion vaccination specifically led to the development of local immunity in the gills, and that the natural route of infection was bypassed by the injection of the pathogen.

Our results show that LFS increases the uptake of substances via immersion, but in accordance with previous authors[79] we also found that longer exposures to substances e.g. flumequine also increases uptake. This suggests that the effects of using LFS can be replaced by longer duration exposures to substances via immersion. However, one needs to consider that the application of LFS in fish is intended for the aquaculture industry, thus the application of LFS can also bring benefits such as: i), a faster and more efficient delivery of substances. ii) A reduced concentration of a given substance is required when administered by immersion. This can be important when the specific substance is expensive or is not available in large amounts (e.g. DNA vaccines, expensive antibiotics or novel compounds under research). iii) For vaccination, besides the increased antigen uptake, ultrasound acts as a physical adjuvant and this has the potential to improve vaccine effectiveness. iv) Application of LFS could exceed the maximum uptake of substances achieved in a conventional bath exposure to a substance. v) As mentioned, changes in permeability achieved by LFS could also be a route of entrance for pathogens, which could be a favourable aspect if the intention is to model experimental infections via immersion.

With regard to the estimation of the effective ultrasound intensity applied in this work, we used an approach similar to [24] who worked with sonophoresis in mammals. How-

ever, in mammals only one transducer is needed for skin sonication, and the distance from the transducer to the skin is constant and at a minimum, which thus allows the reproducibility of treatments under different conditions. In fish, however, LFS is intended for the delivery of substances to a large number of individuals in an ultrasound bath. In this context, the following questions arise: How is the ultrasound intensity applied to an individual fish estimated when there is no constant distance from the transducer; and how may homogeneous sonication be achieved within the ultrasound bath? Frenkel *et al*[29, 80]. and Navot *et al*.[30] handled this problem by applying ultrasound to anesthetized fish fixed to a board. However, this experimental approach is not feasible for large scale vaccination under aquaculture conditions, and does not allow for the assessment of the effect of ultrasound on other tissues. The device used for this study went some way toward solving the "distance" problem by adding a large number of transducers along a narrow canal. However, scaling up this device with a narrow canal will not be the best for handling under practical conditions, and in our calculations, factors which arise from the playback of sound in small tanks such as sound coupling, propagation and reflection were not taken into consideration. As a solution, and to allow better reproducibility of the experiment, we propose that future works should study how to estimate the LFS intensity in the water media.

We present for the first time a technique that can directly help to reduce the use of antibiotics. Incidentally, the drug on which ultrasound had the strongest effect was oxytetracycline, probably the most common antibiotic used worldwide[73]. Thus, our results suggest the potential for substantial reductions in the use of antibiotics in immersion treatments in aquaculture worldwide (Paper III). However, the application of LFS for the reduction of antibiotics still needs to be confirmed in a field trial. Factors such as stress caused by handing prior to the application of LFS and whether the modification to the gill permeability could negatively affect the ongoing infectious process still need to be assessed.

To conclude, we investigated for the first time whether the application of low intensity LFS, that causes minimal or no side effects (Paper I), could enhance the efficiency of immersion vaccination in fish. We showed that LFS can increase the initial uptake of vac-

cines (Paper I and II) and acts as a physical adjuvant in fish (Paper II). The latter was indicated by an increased antibody expression in the gills. In a laboratory vaccination experiment we confirmed the potential of LFS to increase the efficiency of immersion vaccination. Finally, we revealed through the administration of antibiotics, that sonophoresis can also be used for the efficient delivery of other substances by bath immersion (Paper III).

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10 ERKLÄRUNG

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Des Weiteren erkläre ich meine Kenntnisnahme der dem angestrebten Verfahren zugrunde liegenden Promotionsordnung. Ich habe mich anderweitig nicht um einen Doktorgrad beworben und bin nicht im Besitz eines entsprechenden Doktorgrads.

23-02-2015 Berlin,

Cristóbal Cobo

I

Enhanced *Aeromonas salmonicida* bacterin uptake and side effects caused by low frequency sonophoresis in rainbow trout (*Oncorhynchus mykiss*).

Fish & Shellfish Immunology. 2014;36:444-52

Cobo C, Makosch K, Jung R, Kohlmann K, Knopf K.

Abstract

Low frequency sonophoresis (LFS) has been recognized as one of the most advanced technologies in transdermal delivery of substances, due to the modification of the stratum corneum lipid bilayer, in focal skin applications in mammals. Based on these findings, LFS has been suggested as a potential technology to be used for enhancement in immersion fish vaccination. In contrast to mammals where LFS is applied to discrete regions of the skin, in fish the whole individual needs to be exposed for practical purposes. The current study evaluated the impact of LFS at 37 kHz on the uptake of an *Aeromonas salmonicida* bacterin and side effects of the treatment in rainbow trout. Quantitative real time PCR (qPCR) and immunohistochemistry were used to examine the bacterin uptake into skin and gill tissue. Side effects were assessed by behavioural examination, histology and blood serum analysis. The sonication intensity of 171 mW/cm² was enough for increasing skin permeability, but caused heavy erratic swimming and gill haemorrhages. Sonication intensities as low as 105 mW/cm² did not modify skin permeability and enhanced the bacterin uptake into the gill tissue by factor 15 compared to conventional immersion. Following sonication, the gill permeability for the bacterin decreased after 20 min and 120 min by factor 3 and 2, respectively. However, during sonication, erratic swimming of the fish raised some concerns. Further reduction of the sonication intensity to 57 mW/cm² did not induce erratic swimming, and the bacterin uptake into the gill tissue was still increased by factor 3. In addition, a decreasing albumin - globulin ratio in the serum of the rainbow trout within 40 min revealed that LFS leads to an inflammatory response. Consequently, based on both increased bacterin uptake and the inflammatory response, low intensity LFS has the potential to enhance vaccine immunity without significant side effects.

Keywords: Ultrasound, Trout, Vaccination, Aeromonas, Permeability

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II

The adjuvant effect of low frequency ultrasound when applied with an inactivated *Aeromonas salmonicida* vaccine to rainbow trout (*Oncorhynchus mykiss*).

Vaccine. 2015. 33. 1369-1374

Cobo C, Makhutu M, Lumsdon A, Thompson KD, Jung R, Kloas W, Knopf K.

Abstract

Vaccine adjuvants are classified according to their properties of either inducing the persistence of antigens within the animal after immunisation and/or activation of the animal's immune response. The adjuvant effect of low intensity low frequency sonophoresis (LFS) was tested in rainbow trout using an *Aeromonas salmonicida* bacterin vaccine administered by immersion vaccination using LFS at 37 kHz. Quantitative PCR was used to measure bacterial DNA in vaccinated fish up to 35 days post-vaccination, while RT-qPCR was used to assess gene expression during the early and late immune response post-vaccination. Results showed that antigen uptake in the gills was significantly higher in the group exposed to low intensity LFS compared to the other two vaccination groups 15 min post-vaccination, but this initially high uptake did not persist over the rest of the experiment. In the kidney, by comparison, the vast majority of the samples analysed did not show the presence or persistence of the bacterin. Showing that the route of vaccine uptake using the *A. salmonicida* bacterin, does not influence the persistence of the bacterin in the gills or the kidney. On the other hand, LFS induced a higher inflammatory response and T-helper cell activation, characterized by a significant up-regulation of interleukin (IL) 8, IL1ß and CD4, respectively. The expression of IgM, IgT and IgD was up-regulated in gills (being significant for IgM and IgD), but not in the spleen and kidney of the sonicated group. Conversely, IgM was up-regulated in the spleen of the non-sonicated groups, but not in the sonicated group. This highlights that the inflammatory response caused by ultrasound can boost mucosal immune responses, so that ultrasound shows an adjuvant-like effect. It remains to be established whether the up-regulation of IgM, IgT and IgD in gills would be sufficient to offer protection in fish infected with *A. salmonicida.*

Keywords: Sonophoresis, Bacterin, Fish, DIP, Immersion

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III

Application of low frequency sonophoresis and reduction of antibiotics delivered via immersion in rainbow trout (*Oncorhynchus mykiss*).

Manuscript submitted, 2015.

Cobo C, Radinger J, Viehman V, Ariav R, Jung R, Thompson K.D, Kloas W, Knopf K.

Application of low frequency sonophoresis and reduction of antibiotics delivered via immersion in rainbow trout (*Oncorhynchus mykiss*)

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Keywords: Oxytetracycline, florfenicol, flumequine, ultrasound, sonication, rainbow trout

Abstract

A major concern in aquaculture is the use of chemical therapeutics such as antibiotics, because of their impact on the environment as well as on the fish product. As a potential tool for reducing their use, we tested the application of low frequency ultrasound as a method for enhancing antibiotic uptake via the gills and consequently reduce the therapeutic dose required when administered as a bath treatment. For this, rainbow trout juveniles were sonicated and exposed to different concentrations of three commonly used antibiotics (i.e. oxytetracycline, flumequine and florfenicol), administered by bath. Four hours after exposure, concentrations of these substances were measured in the liver and blood of treated fish using UPLC/Q-TOF-MS. Results showed that the ultrasound treatment significantly increased the uptake for all three antibiotics. Indeed, in the uptake of oxytetracycline for example, fish exposed to 20 mg L^{-1},administered with ultrasound, presented even slightly higher concentrations in their liver and blood than fish exposed to 100 mg L^{-1} without sonication. This indicates that sonication preceding a bath treatments can effectively reduce the dose of antibiotics, compared to bath treatment without sonication. Thus, suggests that ultrasound is a strong candidate for reducing the impact of antibiotics on the aquatic environment.

Introduction

Aquaculture is the fastest growing food industry sector [1] and for certain countries it is one of their most profitable export business [2]. However, due to the vast magnitude of this industry in economic terms, and to prevent losses in profit due to several different bacterial diseases, there has been an extensive use of antibiotics in some countries [3]. Indeed, in some cases antibiotics are even applied before the onset of a disease during the production cycle, referred to as "prophylactic treatments" [4-6]. Although the aquaculture industry alleviates the pressure on wild fish stocks as a result of capture fisheries, the industry has been criticized for the large use of chemicals that can impact the surrounding aquatic environment, such as coastal waters, fjords, lakes and rivers [3, 7]. The quantity of antibiotics being applied in aquaculture varies regionally. For example, while Norway uses less than 1 g antibiotics per ton of salmon produced by aquaculture, over 150 g per ton are used in Canada, over 1000 g per ton are used in Chile [8] and more than 700 g per ton of total aquaculture product (indirect estimation) are used in Vietnam [3]. Moreover, in Asian aquaculture 36 different types of antibiotics are currently being used[5].

Generally, antibiotics that are not metabolised or excreted [8] from the fish need to be removed from farm effluents [9, 10]. However, inefficient treatment of wastewater[11], or untreated effluents from fish farms release residues into the aquatic environment. Depending on the type of antibiotic and concentration, such releases can cause changes in the natural aquatic microbial communities, can be toxic to primary producers [12] and increase antimicrobial resistance of pathogens [7, 13]. To reduce the quantity of antibiotics used in aquaculture, approaches such as vaccination have had a dramatic impact on the reduction of antibiotics used to treat particular bacterial diseases [14].

To further reduce the amount of antibiotics used in aquaculture, new technologies such as ultrasound baths appear to be a very promising treatment. Low frequency ultrasound (below 100 kHz) has been recognized to enhance the transdermal delivery of different substances such as estradiol, salicylic acid, corticosterone, and sucrose in mammals [15]. The first studies applying this approach to fish, showed an increase in the permeability of the fish's skin to AgCl particles [16] and BSA [17]. However, negative side effects have been previously reported using ultrasound e.g. erratic swimming and gill

bleeding [18] and even the death of the animal at higher intensities such as 400 mW/cm² [19]. In a more recent study however, we described a safe application of ultrasound, in which only gills permeability appeared modified for only a short period of time [18].

The use of low intensity low frequency ultrasound to improve also the uptake of antibiotics has not yet been investigated in fish. The aim of the present study was to examine the potential of low frequency ultrasound to improve the uptake of three antibiotics commonly used in aquaculture, given by bath immersion, a route commonly used to deliver antibiotics to a commercially important fish species (rainbow trout, *Oncorhynchus mykiss).* For this, the uptake of (i) flumequine (FLU), (ii) oxytetracycline (OTC) and (iii) florfenicol (FLO) were determined by measuring the concentrations of the antibiotics in the liver and blood of treated fish, comparing groups of fish exposed to the antibiotic with and without the ultrasound treatment.

Materials and Methods

Fish husbandry

Rainbow trout that had not been previously treated with antibiotics, ranging from 35 to 70 g, were maintained in a flow through system at a water temperature of 17 ± 1 °C. Fish were fed with a commercial diet (AllerAqua, Christianfield, Denmark) at 0.5 % body weight per day and feeding stopped one day before starting the trial. Water temperature was kept at 17±1 °C and on the trial days water parameters were: 17.6°C, pH 8.1, dissolved oxygen 8.4 mg L^{-1}, (measured with a portable multi-parameter HQ40d (Hach, Loveland, USA).

Chemicals and reagents

Formic acid (LC-MS grade), water (LC-MS grade), tri-chloroacetic acid (TCA, purity ≥ 99%), NaCl (purity ≥ 99.5%), flumequine (FLU, HPLC purity ≥ 98%), oxytetracycline hydrochloride (OTC, HPLC purity ≥ 95%), florfenicol (FLO, TLC purity ≥ 98%) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Methanol (MeOH, LC-MS grade), acetonitrile (MeCN, LC-MS grade), dimethyl sulfoxide (DMSO, purity ≥ 99.5%) from Carl Roth (Karlsruhe, Germany) and SPE Quaternary Amine from BakerBond (Philippsburg, USA). Stock solutions of the antibiotics were freshly prepared for the trial, dissolving 150 mg of FLU in 500 µl ml sodium hydroxide 1 M, OTC was directly dissolved in aquarium water and 60 mg of FLO dissolved in 100 µl DMSO (Carl Roth).

Treatment with ultrasound and antibiotics

Exposure of fish to low frequency ultrasound was performed in a long shaped bath with a capacity of 17 L (Fig. 1). Each lateral wall of the bath was equipped with 20 ultrasonic transducers, which provide low frequency ultrasound at 37 kHz. To determine the applied ultrasound intensity administered to the fish, (i) the overall power consumption of the transducers, (ii) the area of the side walls and (iii) an efficiency coefficient of 0.7 were considered [20] using the following equation: Intensity (W/cm²) = power (W) / area (cm²) x 0.7

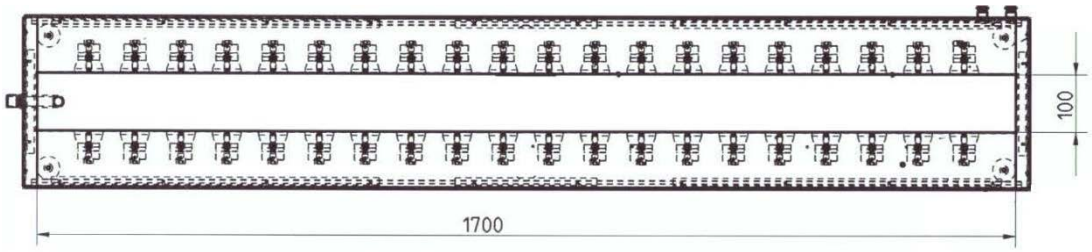

Figure 1: Schematic top view on the ultrasound device, illustrating the assemblage of transduces on the side walls of the bath. Dimensions of the bath in mm.

The fish were exposed to three different antibiotics at two different concentrations by bath immersion administered with or without ultrasound. Twelve groups (four groups per antibiotic, n=7 per group) were used in total. Fish were carefully netted into the sonication bath in a randomized order and sonicated at an intensity of 64 mW/cm². This intensity was chosen to reduce side-effects, while still increasing permeability of the gills as previously shown for vaccine uptake [18, 21]. The length of sonication was five minutes as recommended by Zhou [22] and applied in five pulses of 30 s ON/OFF i.e. a total of 2.5 min of net sonication time. Fish that were not sonicated were also kept in the sonication bath for 5 min, but with the device remaining off. Thereafter, each group was exposed either to FLU (10 mg L^{-1} or 20 mg L^{-1}), OTC (20 mg L^{-1} or 100 mg L^{-1}) or FLO (2 mg L^{-1} or 10 mg L^{-1}) in 5-L buckets with constant aeration for 1 h.

After exposure to the antibiotics, the fish in each group were transferred to a single flow through aquaria (100 L) for 4 h. The fish were then euthanized with an overdose of MS-222, and blood sampled from the caudal vein of fish. Samples were kept overnight at 4 °C before collecting the serum by centrifugation at 9,000 g for 5 min. This was then stored at -80°C. The liver was removed, frozen in liquid nitrogen and stored at -80 °C until analysed.

<u>Antibiotic analysis by ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry (UPLC/Q-TOF-MS)</u>

Extraction and quantification of FLU, OTC and FLO from the livers and serum of fish, sampled as described above, was performed. For samples from fish exposed to FLU, a solid-liquid extraction using MeCN was performed as previously reported[23]. Liver samples were thawed and weighted, then 0.1 % (v/v) formic acid in MeCN (FA/MeCN) was added and the tissue lysed in a tissue lyser (Qiagen, Hilden, Germany). For the serum

samples, 60 µl of the sample and 50 µl TCA (with MeCN+FA) was added to eliminate the protein content. The samples were then centrifuged (3 min at 2300 g) and impurities removed by adding ~5 mg of NaCl and Amino-Phase (BakerBond). Collected supernatants were evaporated until dry on a heater block (40°C) under a stream of nitrogen gas. The samples were resuspended in 200 µl of MeCN+FA and transferred to an HPLC vial (Agilent Technologies, Palo Alto, CA, USA).

Extraction procedures for OTC and FLO were conducted using MeOH, as base on previously described protocols [24-27]. The extraction was carried out on ice and using only glass apparatus. Liver was weighted and homogenized manually in Elvehjem glass potters with 500 µl MeOH + 0.1% Formic acid (FA/MeOH), and the homogenate was transferred to a glass tube. Thereafter, samples were centrifuged (3 min at 660 g) and supernatants transferred to an HPLC vial (Agilent Technologies). For serum, 120 µl of the sample was taken and 50 µl of TCA + 1000 µl of FA/MeOH added. Samples were centrifuged (3 min at 2300 g) and supernatants transferred to an HPLC vial and measured immediately.

The liquid chromatography was performed using an UPLC Agilent 1290 Infinity equipped with an Agilent Eclipse Plus C18 2.1 x 50 mm, 1.8 µm column. The measurements were made using a gradient flow rate of 400 µl min^{-1} for FLU column maintained at 30°C, and for the OTC and FLO columns were maintained at 20°C, 1µl of sample was injected onto the column. For the Q-TOF, an Agilent 6550 iFunnel Q-TOF LC/MS was used, with a Dual AJS ESI positive ion source. Finally, data acquisition and analysis for all three antibiotics was performed using a MassHunter Qualitative and Quantitative software (Agilent Technologies). Since the complete liver was homogenized, the value obtained was expressed as a concentration per milligram of tissue.

The ranges of detection were as follows: FLU 10 – 250 ng ml^{-1}, OTC 5 – 100 ng ml^{-1} and FLO 25 – 250 ng ml^{-1}. If the detection of the substance was lower than the standards, samples were considered positive, but not quantified. In the case of no peaks or unclear spikes, the samples were considered negative.

Statistics

For FLU and OTC, the effects of ultrasound application on the measured uptake were assessed by a full-factorial ANOVA, using the factors ultrasound exposure (yes or no), exposure dose (for FLU 10 or 20 mg L^{-1} and for OTC 20 or 100 mg L^{-1}) and tissue (blood or liver). Student T-tests were performed to compare values between the different groups. Normal distribution within single groups was confirmed before using Shapiro-Wilk tests ($p > 0.05$). For OTC, the serum samples of the group exposed to 20 mg L^{-1} and no ultrasound were below the quantifiable limit, but considered positive (see previous section). Thus, a threshold value of 4.9 ng ml^{-1} (just below the detection limit) was assigned for these samples. The detected concentrations of one group (OTC in liver at 100 mg L^{-1} without ultrasound treatment) were not normally distributed (Shapiro Wilk test, $p = 0.027$), which could not be improved by data transformation. Still we considered a full-factorial ANOVA also for OTC based on the reported robustness of the ANOVA against violation of normality [28]. For FLO, a Mann Whitney *U*-test and descriptive statistics were applied. Since an increased uptake was only expected after sonication and at higher doses, one sided tests were performed throughout. Significance was assumed for $p \leq 0.05$. All analyses were performed using the statistical software SPSS 22 (SPSS Inc. Chicago, Illinois, USA).

Results

Oxytetracycline

The highest concentration of OTC found in liver and serum (284 ng mg^{-1} and 312 ng ml^{-1}, respectively) was in the group exposed to 100 mg^{-1} of the antibiotic after the ultrasound treatment. In contrast, the group exposed to 20 mg L^{-1} without ultrasound had the lowest concentration with 117 ng mg^{-1} and 9.5 ng ml^{-1} measured in liver and serum, respectively. Full factorial analysis revealed significantly higher uptake when fish were exposed to ultrasound (ANOVA, F_1= 23.02 p< 0.001). OTC uptake in both liver and serum was significantly higher after ultrasound exposure (ANOVA, F_1= 4.40 p= 0.023 and F_1= 28.90, p< 0.001, respectively). The group exposed to 20 mg^{-1} with ultrasound was not significantly different from the group exposed to 100 mg^{-1} without ultrasound for uptake in liver and in serum (t-Test t_{11}< 0.01; p= 0.482 and t_{12}= 2.11, p= 0.169, respectively) (Fig. 2).

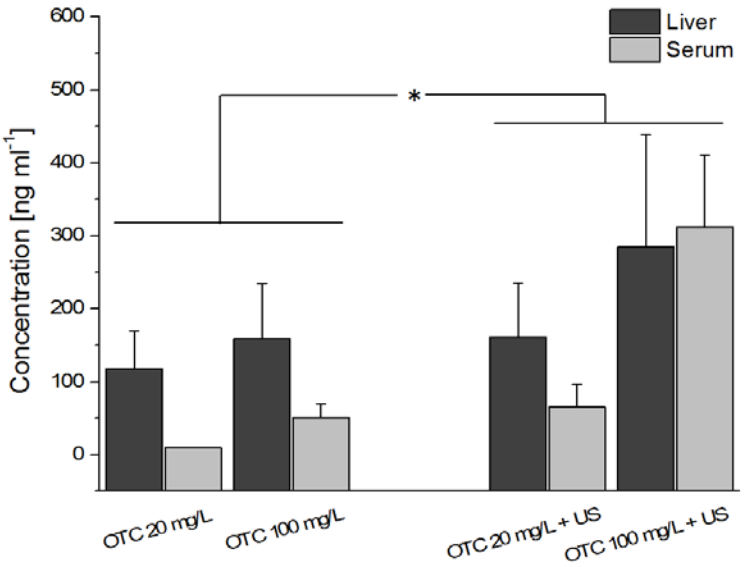

Figure 2: Mean concentration and standard deviations (n=7) of oxytetracycline (OTC) in the liver and serum of rainbow trout after exposure to OTC at 20 mg L^{-1} or 100 mg L^{-1} by immersion with or without ultrasound (US). An Anova significant difference is marked by an asterisk.

Flumequine

The group treated with ultrasound and then exposed to FLU at 20 mg L^{-1} showed the highest concentrations, presenting 1394 ng mg^{-1} in liver and 404 ng ml^{-1} in serum. The lowest detected concentrations were found in the liver of the fish exposed to 10 mg L^{-1} without the ultrasonic pre-treatment (762 ng mg^{-1}), and in the serum of the fish exposed to 20 mg L^{-1} without ultrasonic pre-treatment (268 ng ml^{-1}). The groups exposed to 10 mg L^{-1} with ultrasound and 20 mg L^{-1} without ultrasound showed very similar concentrations (liver: 896 ng mg^{-1} and 851 ng mg^{-1}, serum: 348 ng mg^{-1} and 268 ng ml^{-1} respectively). Overall, the uptake of FLU was significantly higher when fish were exposed to ultrasound (ANOVA, F_1= 4.09, p= 0.024). A split analysis for effects of ultrasound revealed a significantly higher uptake in liver (ANOVA, F_1=2.93, p=0.049) and for a concentration of 20 mg^{-1} (ANOVA, F_1=4.48 p=0.022). Comparisons between the groups treated with 10 mg^{-1} with ultrasound and 20 mg^{-1} without ultrasound did not reveal significant differences between the groups (t-Test; t_{13}= 0.51, p=0.434 and t_{13}= 1.30, p= 0.154 for liver and blood, respectively) (Fig. 3).

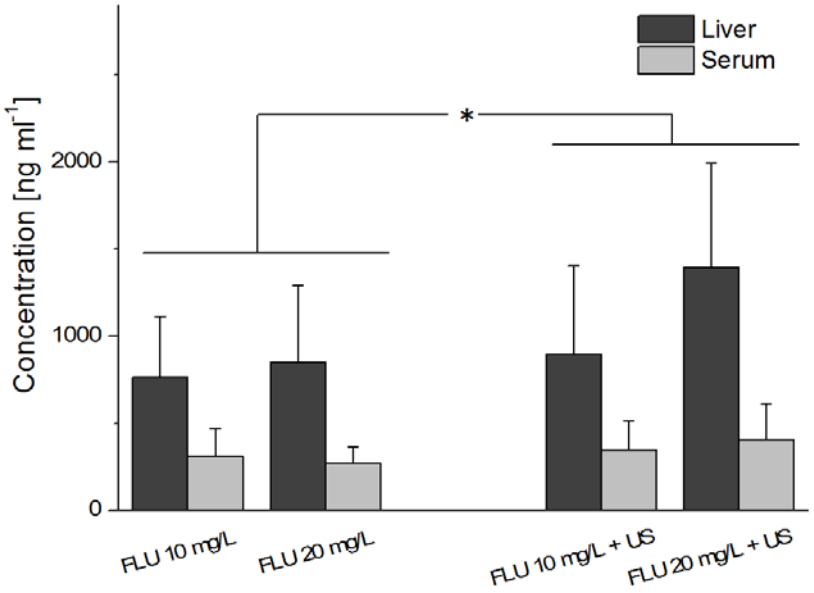

Figure 3: Mean concentration and standard deviations (n=7) of flumequine (FLU) in the liver and serum of rainbow trout after exposure to FLU at 10 mg L-1 or 20 mg L-1 by immersion with or without ultrasound (US). An Anova significant difference is marked by an asterisk.

Florfenicol

Fish with ultrasonic treatment exposed to 10 mg L^{-1} showed the highest concentration in their liver (1307 ng mg^{-1}). The liver samples of fish exposed to 2 mg L^{-1} without ultrasound were all negative. In contrast, six out of seven liver samples were positive for the fish exposed to the same concentration (2 mg L^{-1}) with ultrasound. FLO concentrations in liver samples of fish exposed to 10 mg^{-1} with ultrasound compared to samples without ultrasound, were significantly different (Mann Whitney *U*-test; U= 2.00, p= 0.002). For serum, only three samples from all groups were positive, belonging to the group exposed to ultrasound and 10 mg^{-1} FLO (Fig. 4).

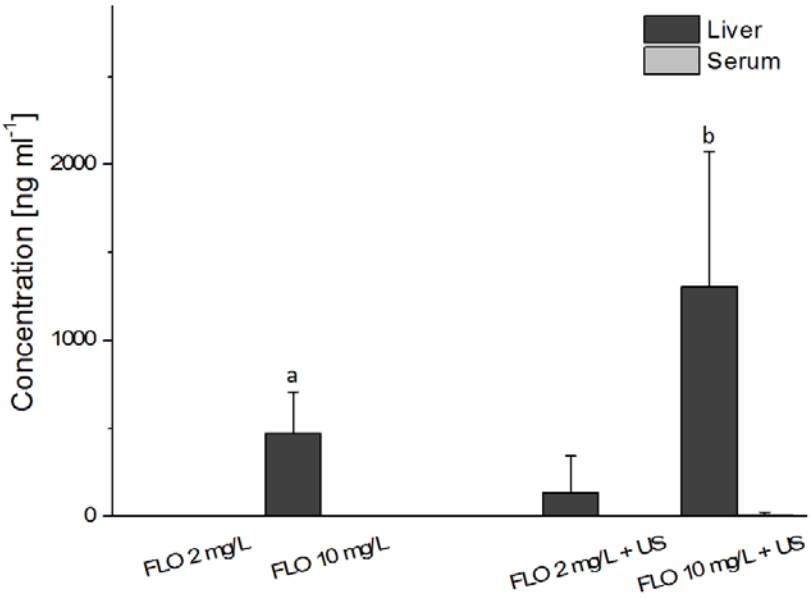

Figure 4: Mean concentration and standard deviations (n=7) of florfenicol (FLO) in the liver and serum of rainbow trout after exposure to FLO at 2 mg L-1 or 10 mg L-1 by immersion with or without ultrasound (US). Significant differences in Mann Whitney U-test pair wise comparisons are marked with consecutive letters. No other analyses were performed due to missing values.

Discussion

In the present study we could demonstrate that ultrasonic pre-treatment of fish can significantly increase the uptake of the three antibiotics commonly used in aquaculture against bacterial diseases [3, 29]. As previously shown, ultrasound was safely applied to rainbow trout [18] and the present study reveal that sonication can increase the efficiency of therapeutic treatment administered by bath, by a factor of five and two for OTC and FLU, respectively. The ultrasound also increases the uptake of FLO, however we could not determine the magnitude of the uptake. This detected increase in uptake suggests that ultrasound is a very useful technique for treating farmed fish with antibiotics and offer for the first time a potential direct instrument to lessen the environmental impact of antibiotic usage.

The underlying mechanisms involved in the interaction of low frequency ultrasound with biological membranes have been described previously[20]. A drop in the atmospheric pressure of water causes cavitation in the water medium and/or oscillation on the cell lipid bilayer membranes[30]. When the oscillations occur in biological tissue an increase in permeability within the tissues results, alternatively cavitation can also cause lysis of the outer epithelia and create an increase in permeability [31]. The oscillations can be considerable attenuated in multi-layered tissues. Thus, single cell epithelium layers, such as gill lamellae, are more sensitive to the effects of low frequency ultrasound compared to the multi-layered epithelia of the skin [18]. Consequently, the increased uptake of antibiotics observed in the present study with low ultrasonic intensities is mainly attributed to changes in the permeability of the gills rather than to changes of the skin permeability [32].

The greatest effect of ultrasound on the uptake of the three analysed antibiotics was observed for OTC, which is a semi-lipophilic drug and one of the most commonly used antibiotics used in aquaculture globally[33] . As previously reported for mammals, ultrasound enhances the transdermal delivery of hydrophilic compounds by formation of aqueous channels after the ultrasonic treatment [15]. This could be a reason why ultrasonic treatment enhanced the uptake of OTC hydrochloride much more than the uptake of FLU and FLO, which are both lipophilic substances.

With the present results, we suggest that sonication can reduce antibiotics usage in aquaculture by reducing the concentration required in the bath treatment. However,

an alternatively option for the reduction could be to reduce the total number of therapeutic treatments applied. This could be achieved since OTC has a long elimination half-life dose[34] and groups with sonication treatment and exposed to the "high" concentration of the substances showed higher concentrations in tissues than without sonication. Then an effective concentration dose can be maintain for a higher period of time.

As showed by others, the uptake of FLU was detectable after a bath administration [35]. This is in contrast to a few reports in which FLO and OTC uptake is minimal or not detectable after a bath administration[36-38]. The reason why these substances were detected in the study, could be that (i) liver, as well as blood, was analysed in the study, (ii) the detection limits were lower than used in the other studies or (iii) the water temperature was slightly higher in this trial compared to previous reports, thus favouring the uptake of the antibiotics. It has been previously reported that no detectable levels of FLO were evident in the blood of koi carp given a bath treatment with 80 mg L^{-1} FLO [38]. In contrast, in the present study FLO accumulation was detected in the liver after exposure to only 10 mg L^{-1}, showing that the liver is a more suitable organ to assess the uptake of lipophilic substances.

Finally, the results show the potential of ultrasound technology for the treatment of fish in aquaculture by making antibiotic bath treatments more efficient and thus reducing the environmental impact and economic costs associated with medicating the fish. In association with appropriate prophylactic measures such as vaccination and improved biosecurity, this technique can contribute to an appreciable reduction in the use of antibiotics used in aquaculture.

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